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Sequence Homologies between *Mycoplasma* and *Chlamydia* spp. Lead to False-Positive Results in Chlamydial Cell Cultures Tested for Mycoplasma Contamination with a Commercial PCR Assay▽

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Mycoplasma contamination is a frequent problem in chlamydial cell culture. After obtaining contradictory contamination results, we compared three commercial PCR kits for mycoplasma detection. One kit signaled contamination in mycoplasma-free *Chlamydia pneumoniae* cultures. Sequencing of cloned PCR products revealed primer homology with the chlamydial genome as the basis of this false-positive result.

Contamination of cell cultures with oral *Mycoplasma* species such as *M. fermentans* or *M. hyorhinis* alters host cell trafficking and shows profound inhibitory effects on infectivity and growth of the obligate intracellular chlamydiae *Chlamydia pneumoniae* and *Chlamydia trachomatis* (1). Thus, working with chlamydiae requires regular monitoring of cell lines and primary cells for contamination, which is usually done with commercial PCR kits manufactured for detecting mycoplasmas in cell cultures. False-negative as well as false-positive results in such tests have deleterious consequences for the validity of research results as well as for the sensitivity of chlamydial diagnostic recovery. Contradictory results between PCR assays and the observed unaltered *C. pneumoniae* growth characteristics, which gave no indication of mycoplasma contamination (as compromised chlamydial growth or altered biological behavior), led us to compare three frequently used commercial mycoplasma PCR kits: Venor GEM (targeting 16S rRNA genes; Minerva Biolabs, Berlin, Germany), the nested PCR mycoplasma detection set (targeting 16S and 23S rRNA genes; TaKaRa Bio Inc., Otsu, Shiga, Japan), and the MycoTrace assay (target not given; PAA Laboratories, Dartmouth, MA), which is advertised to detect considerably more mycoplasma species than do other assays. Chlamydial taxonomy has recently been clarified by dropping the genus "*Chlamydophila*" and restoring "*Chlamydia*" in *C. pneumoniae*, which we use in this report (3).

Culture samples from the vascular *C. pneumoniae* isolate CV6 (4), two *C. trachomatis* isolates of serovars A (ATCC VR-571B) and D (ATCC VR-885), uninfected epithelial host cell line HEp-2 (ATCC CCL-23), primary human coronary artery endothelial cells (HCAEC), and primary human coronary artery smooth muscle cells (CASMC) were subjected to the PCR assays from Minerva, PAA, and TaKaRa, which were all conducted according to the instructions supplied. PCR products were analyzed on the Experion automated electrophoresis system (Bio-Rad, Hercules, CA). All analyses were repeated three times in triplicate. In all samples containing *C. pneumoniae*, we consistently obtained a mycoplasma-positive result with the PAA MycoTrace assay but negative results with the Venor GEM and TaKaRa assays. Uninfected cells and *C. trachomatis* cultures tested negative for mycoplasma in all

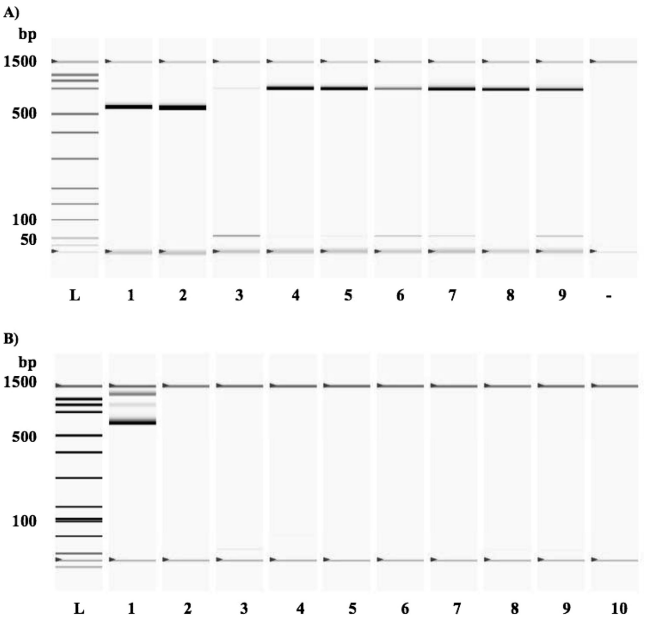

FIG. 1. Gel electrophoresis of products from PAA MycoTrace (A) and TaKaRa (B) PCR amplification of *C. pneumoniae* cell cultures. (A) Lanes 1 and 2, uncontaminated *C. pneumoniae* samples with "mycoplasma-positive result" (amplicon at 520 bp); lanes 3 and 4, HEp-2 cell samples; lane 5, coronary artery smooth muscle cells; lane 6, coronary artery endothelial cells; lanes 7 and 8, *C. trachomatis* serovars A and D, respectively; lane 9, PCR-negative buffer control. Amplified internal control is present in lanes 3 to 9 at 700 bp. (B) Lane 1, kit positive control, at 810 bp from the first PCR and at 590 bp from the nested PCR; lanes 2 and 3, uncontaminated *C. pneumoniae*; lanes 4 and 5, HEp-2 samples; lane 6, coronary artery smooth muscle cells; lane 7, coronary artery endothelial cells; lanes 8 and 9, *C. trachomatis* serovars A and D, respectively; lane 10, PCR-negative buffer control. An internal control is not provided.

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Sense Primer Alignments

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Mycoplasma primer: CGC CTG GGT AGT ACA TTC GC
                   ||| |||  |  ||| |||  || ||
C. pneumoniae:     CGC CTG AGG AGT ACA CTC GC
                   ||| |||  |  ||| |||  || ||
C. trachomatis:    CGC CTG AGG AGT ACA CTC GC
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Antisense Primer Alignments

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Mycoplasma primer: GCG GTG TGT ACA AGA CCC GA
                   ||| ||| ||| ||| ||  ||| ||
C. pneumoniae:     GCG GTG TGT ACA AGG CCC GA
                   ||| ||| ||| ||| ||  ||| |
C. trachomatis:    GCG GTG TGT ACA AGG CCC GG
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FIG. 2. Sequence alignment between a cloned primer pair of the PAA MycoTrace assay, *C. pneumoniae*, and *C. trachomatis* 16S rRNA genome sequences.

three assays. Figure 1 shows the PAA MycoTrace and TaKaRa assay results in comparison.

We then used mupirocin treatment of *C. pneumoniae*-infected cells in an established and effective procedure for elimination of mycoplasma infection (2). However, the PAA MycoTrace assay kept yielding its mycoplasma-positive signal even after this antimycoplasmal treatment. Biological or microscopical signs of mycoplasma contamination were completely absent in the cultures. Therefore, we decided to analyze the PAA MycoTrace assay primers and amplicons for potential homologies with *C. pneumoniae* genome sequences by cloning the PCR products. PCR amplicons that resulted from *C. pneumoniae* cell cultures were cloned using the QIAquick PCR purification kit and the Qiagen PCR Cloning^{Plus} kit (Qiagen, Hilden, Germany), which contains competent cells. Twelve clones from different agar plates were selected and propagated. Plasmid DNA was purified with a plasmid purification kit (Macherey-Nagel, Düren, Germany). Digestion with EcoRI (Promega, Madison, WI) for 2 h at 37°C followed in order to control for integration of PCR products. Eight clones showed an insert of the expected size of about 500 bp. These eight clones, which represented the sequences associated with the expected primer regions, were sequenced with the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Carlsbad, CA). Four clones had an unusable insert.

Sequence alignment showed extensive homology of the expected primer regions from the PAA MycoTrace assay with *C. pneumoniae*. The clones had a 520-bp size with minor nucleotide exchanges at the 5′ and 3′ ends. Apparently, the manufacturer uses a mixture of primer pairs to extensively cover mycoplasma species, which unfortunately leads to false-positive results when *C. pneumoniae* cultures are examined. Figure 2 shows alignments of a cloned mycoplasma primer pair and 16S rRNA chlamydial genome sequences (Fig. 2). The chlamydial sequence targets shown occur in all four *C. pneumoniae* genomes submitted to GenBank (e.g., in GenBank AE009440.1 and *C. pneumoniae* TW-183) as well as in the *C. trachomatis* genomes (e.g., GenBank CP002054.1 and *C. trachomatis* D-LC). With *C. pneumoniae*, the mycoplasma primers deliver a 520-bp amplicon. However, our two *C. trachomatis* isolates always tested negative in the PAA MycoTrace assay under the manufacturer's test conditions in spite of the extensive homology seen in the available sequences. The single mismatch at the 3′ end of the antisense primer may help prevent amplification.

Uphoff and Drexler (7) originally suggested a mycoplasma detection protocol using 9 different primers which all target 16S rRNA genes. Four of these indeed have 100% identity to sequences of our clones and are thus apparently the base for the false-positive results of the PAA MycoTrace assay when *C. pneumoniae* DNA is present in the reaction mixture. The TaKaRa assay, on the other hand, did not produce false-positive or false-negative signals but lacks an internal control. Designed as a nested PCR, this assay is also quite time-consuming. The Venor GEM assay, while easier in handling, commonly shows inhibition of the internal control in our hands and may thus produce false-negative results (data not shown).

Mycoplasma contamination of cell cultures and chlamydial strains is frequent enough and one of the major obstacles in research on host-pathogen molecular interaction and in recovering and propagating new chlamydial isolates. In this respect, a false-positive mycoplasma result has the same deleterious consequences that result from true contamination, namely, loss of strains and data. Extensive homology was found within the mycoplasmal and chlamydial 16S rRNA gene. The importance of primer design when establishing assays for detecting mycoplasma contamination is in fact well known (5, 6), and manufacturers should advise their customers on conditions under which primer specificity may be compromised. In conclusion, it is problematic to use the PAA MycoTrace assay for detection of mycoplasma contamination in *C. pneumoniae* cell cultures due to sequence homologies in the mycoplasma primer pairs that will invariably lead to false-positive results. No reactivity was found with *C. trachomatis* serovars A and D. However, it might prove helpful to pretest the specificity of the PAA MycoTrace assay when applying it to *C. trachomatis* cultures as extensive sequence homologies to mycoplasma species exist here, too. When applied to nonchlamydial cell cultures, there should be no issues with the specificity of this assay.

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